Sensitivity of field isolates of *Eimeria acervulina* and *E. maxima* from three regions in Brazil to eight anticoccidial drugs

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ABSTRACT Rotation with different active ingredients is among the most effective and recommended strategies to preserve the efficacy of anticoccidial drugs and reduce the emergence of resistance. Tools such as anticoccidial sensitivity tests (**ASTs**) are ideally used to make rational rotation programs and bring benefits to production. The objective of this study was to evaluate the sensitivity of *E. acervulina* (**EA**) and *E. maxima* (**EM**) from 3 different regions in Brazil, by using four ASTs. Feces samples weighing 6 to 7 kg were collected in the regions of São Paulo, Paraná, and Minas Gerais. Prevalent oocysts from feces were filtered, identified, and quantified to conduct 2 ASTs with EA and 2 with EM. The same experimental design was used in every AST (4 replicates per treatment, with 6 birds each, for a total of 240 birds). Treatment groups were a nonchallenged and nonmedicated control group (T1), a challenged and nonmedicated control group (T2), and the other groups challenged and treated with the following compounds: lasalocid (90 ppm − T3), maduramycin (6 ppm − T4), decoquinate (30 ppm − T5), nicarbazin +semduramicin (66 ppm − T6), monensin (110 ppm − T7), salinomycin (66 ppm − T8), narasin+nicarbazin (100 ppm − T9), and nicarbazin (125 ppm − T10). At the end of each AST (20 d), the percent change (delta value) between the treated group (T3 to T10) and the control group (T2) was calculated for the following variables: body weight gain, feed conversion ratio, lesion score, and an indicator of percentage of optimal anticoccidial activity (**POAA**) that included T2. Different sensitivity levels of EA and EM isolates could be identified. As a whole, drugs from T5 and T3 groups showed higher delta values when compared to other compounds, whereas the lowest sensitivity levels of these isolates were observed in groups T4 and T7. Despite some limiting factors, ASTs can be a good tool for strategic selection of anticoccidial drugs in order to maintain efficacy and extend the lifespan of these molecules.

Key words: coccidiosis, anticoccidial sensitivity test, broiler chicken, anticoccidial resistance, anticoccidial drug

INTRODUCTION

Coccidiosis control should be among the pillars of poultry health to achieve better production results. Recent financial estimates suggest that the costs involved with prophylaxis, treatment, and production losses due to coccidiosis can amount to around U$14 billion worldwide a year (Blake et al., 2020a). More than 80% of the drugs for coccidiosis control were introduced between 1948 and 1980. The first cases of resistance were described from one to 11 yr later, for a mean of 4.2 yr (Chapman, 1997; Noack et al., 2019). Most of these drugs have been in use since then, thus leading to many assessments of resistance or loss of sensitivity (Yadav and Gupta, 2001; Conway et al., 2001; Peek and Landman, 2003; Bafundo et al., 2008; Arabkhazaeli et al., 2013; Zhang et al., 2013; Lan et al., 2017).

Nonstop use of anticoccidials can potentially lead to gene modification in *Eimeria* spp. Nevertheless, practical molecular methods are still unavailable to evaluate these changes (Blake et al., 2020b). Therefore, anticoccidial sensitivity tests (**ASTs**) need to be performed in vivo, consequently narrowing the choice of different drugs and *Eimeria* strains for testing (Peek and Landman, 2011). The parameters evaluated in ASTs may

vary among lesion score (Johnson and Reid, 1970; Mcdougald et al., 1987), oocyst multiplication by OPG (Lan et al., 2017; Chasser et al., 2020), performance parameters such as food conversion ratio (**FCR**), body weight gain (**BWG**), and mortality (Stephan et al., 1997; Chapman, 1998), or the combination of several parameters in formulas generating indexes (Stephan et al., 1997; Arabkhazaeli et al., 2013; Lan et al., 2017).

In Brazil, there has been very little use of ASTs. In contrast, in the United States and Europe, due to a greater availability of resources and the close relationship between universities and the pharmaceutical industry, these ASTs are performed quite often to guide the selection of anticoccidial drugs, however, they are rarely published in literature. ASTs also represent the most feasible tool to assess these drugs' efficacy in product registration or extension trials required by regulatory authorities (Chapman, 1997; Naciri et al., 2003; Peek and Landman, 2003).

Regardless of the method chosen to assess sensitivity to anticoccidials, it is important to bear in mind what most impacts each broiler chicken company's environment and to strive for a cost-benefit balance between what will bring higher returns, welfare, and sustainability to that business. In modern poultry production, effective coccidiosis control is related to the correct use of available tools. In this sense, good rearing practices and prophylactic drugs are the first line of action, followed by vaccines and alternative products (Blake et al., 2017; Fatoba and Adeleke, 2018).

Considering human population growth and the increasing need for poultry meat to provide dietary protein, anticoccidials have been playing, and will continue to play, a fundamental role in coccidiosis control, the enhancement of poultry health and welfare, the effectiveness of production, and economic and environmental sustainability (Kadykalo et al., 2018).

It is important to know the sensitivity level of the different field *Eimeria* spp. in each broiler chicken company in order to make more assertive decisions regarding rotational management of anticoccidials. This is done to maintain the available compounds' efficacy and achieve optimal rates of coccidiosis prevention and productivity gain. The objective of this study was to evaluate the sensitivity of *E. acervulina* and *E. maxima* from three different regions in Brazil to the compounds chiefly used locally by means of 4 ASTs.

MATERIALS AND METHODS

Ethics Statement

All procedures involving animals were conducted according to the standards of the Committee on Ethics for the Use of Animals (**CEUA**, acronym in Portuguese), at the Centro de Amparo à Pesquisa Veterinária (Veterinary Research Support Center). AST 1 Protocol: 0003/2018; AST 2 Protocol: 006/2019; AST 3 Protocol: 0009/2020; AST 4 Protocol: 0010/ 2020.

Sampling and Origin of Samples

Samples were collected at 3 broiler chicken companies in 3 Brazilian states on different dates: São Paulo (2018), Paraná (2019), and Minas Gerais (2020). The 2020 samples were submitted to 2 ASTs: one with *E. acervulina* isolates and the other with *E. maxima* isolates. In this study, the term AST refers to the complete set of parameters evaluated, not only to lesion scores.

AST 1 Approximately 6 kg of feces were collected at a broiler chicken company in the state of São Paulo in September 2018. The samples were collected at 8 different poultry farms, where *E. maxima* oocysts were most prevalent. This company did not report the anticoccidial program used at the time of sampling.

AST 2 Approximately 7 kg of feces were collected at a broiler chicken company in the state of Paraná in September 2019. The samples were collected at 12 different poultry farms, where *E. acervulina* oocysts were prevalent. The shuttle anticoccidial program was nicarbazin + narasin (1−21 d) and monensin (22 d until 5 d prior to slaughter) at the time of sampling, as reported by the broiler chicken company.

AST 3 Approximately 7 kg of feces were collected in February 2020 at a broiler chicken company in the state of Minas Gerais. The samples were from 6 different poultry farms with a high prevalence of *E. acervulina* and *E. maxima*. Therefore, it was decided to conduct an AST for each species of *Eimeria*. In this case, it was for *E. acervulina*. According to this company, the shuttle anticoccidial program at the time of collection was nicarbazin + semduramicin (1−21 d), and salinomycin (for 22 d up to slaughter).

AST 4 The same isolate as in AST 3. However, *E. maxima* oocysts were set aside for this experiment.

Feces Collection, Inoculum, and Identification of Eimeria Species

The selection of poultry farms was based on their history of coccidiosis challenge in previous flocks (presence of lesions observed in necropsy indicative of the target *Eimeria* species) informed by the company's veterinarian. It was also confirmed on the spot by necropsy just before collection. The flock age ranged from 21 to 35 d. Fresh feces samples, free of litter and cecal content, were collected at several places in the poultry houses on each farm. After collection, the feces samples were immediately placed in a potassium dichromate solution, at the rate of 100 mL solution for 900 g feces. The solution consisted of 2.5 g potassium dichromate diluted in 97.5 mL distilled water. Samples were placed in polyethylene terephthalate bottles at room temperature and forwarded to a laboratory within three days of collection.

Oocysts present in the feces were filtered and microscopically identified (by size). This was then confirmed by a pre-test to determine the appearance and location of the lesions (Long and Reid, 1982). The filtration procedure was conducted to separate a single target species for the in vivo test, although other oocysts present in the

sample may have passed the filtering process. The large amount of feces collected made it possible to achieve the number of oocysts to each AST with no need to propagate them in live chickens. Obtained oocysts were quantified in the final volume.

To assess virulence and dose of *Eimeria* before each AST, a pre-test was carried out with different oocyst doses. Thirty-5 birds were included in the pre-test. They were divided into 7 groups of 5 birds each. Each group received a dilution of the original inoculum, according to the total volume of the isolate, at the age of 14 d. The lesion score was evaluated (Johnson and Reid, 1970) in all birds at 20 days of age (6 d postinoculation). The group in which at least 80% of the birds showed lesion score ≥2 and 20% weight gain reduction, with minimal mortality, was elected as the infecting dose for the AST (Mcdougald et al., 1987).

Birds and Experimental Design

One-day-old male Cobb 500 broiler chicks were housed in *Eimeria*-free suspended cages. They received feed and water ad libitum throughout the experimental period. The same experimental design was used in the four ASTs. A total of 240 broilers were included and divided into 10 treatments of 4 replicates, with 6 birds each (Table 1). The birds received a (nonmedicated) standard starter feed up to 12 d of age and then medicated feed with the respective anticoccidial up to 20 d of age (Lan et al., 2016).

All birds from treatment groups T2 to T10 were inoculated with 1 mL of *Eimeria* oocyst solution at 14 d of age (concentrations were AST 1: 100,000 *E. maxima* oocysts/bird; AST 2: 150,000 *E. acervulina* oocysts/bird; AST 3: 80,000 *E. acervulina* oocysts/bird; AST 4: 25.000 *E. maxima* oocysts/bird) orally (gavage). Treatment group T1 (negative control) received 1 mL of distilled water, also by gavage.

Every bird and all leftover feed were weighed at 20 d of age for weight gain and feed conversion ratio calculations. All birds were euthanized on the same day, and coccidiosis lesion scoring was performed by using a 0 to 4 scale (Johnson and Reid, 1970). Percentage of Optimum Anticoccidial Activity (**POAA**) was calculated based on weight and mortality data. POAA = (SGR in the medicated group [T3 to T10] − SGR in the challenged and nonmedicated group [T2]) / (SGR in the nonchallenged and nonmedicated group [T1] − SGR in the challenged and nonmedicated group [T2]) × 100%, where SGR (Survival and Growth Rate) was defined as the final weight (all birds in the cage + weight of the dead birds) divided by the initial weight (prechallenge) (Rathinam and Chapman, 2009; Lan et al., 2017). The *Eimeria* isolates were considered resistant (**R**) if the POAA was ≤50%, partially resistant (**PR**) if the POAA was between 51%-74%, and sensitive (**S**) if the POAA was ≥75% (Rathinam and Chapman, 2009).

Evaluation of Drug Sensitivity

To analyze the results of these four experiments, they were combined according to the challenge (*E. acervulina* or *E. maxima*). All performance data were described as a delta value of experimental group (T3 to T10) in relation to the positive control group (T2) for the parameters BWG, FCR, and lesion score (**LS**). The delta was calculated by performance (FCR, BWG, and LS) of experimental group minus positive control group, divided by performance of experimental group, multiplied by 100. Hence, when percentual delta is positive, it means that experimental group was numerically superior and when negative, numerically inferior to the positive control group. Note that a positive delta does not mean necessarily better result, for example, FCR a positive delta means that FCR of experimental group was superior that positive control. This is not a good result, but remains numerically superior. POAA data were described according to the result generated by the formula itself.

Statistical Analysis

To assess the influence of the use or nonuse of the anticoccidial drugs, percentual delta of FCR, BWG, LS, and POAA variables were combined by meta-analysis models. For each experimental group of each AST, a pooled effect was calculated. Inverse variance for the mean of continuous measures was used to obtain the pooled effect. This pooled effect represents a weighted mean of all values and a confidence interval of 95% is presented considering variability intragroup and inter-group. This means that is not a simple weighted mean but can be interpreted as well. Weight of each value was defined by proportion of their inverse variance as described by

Table 1. In feed anticoccidial treatments included in the four anticoccidial sensitivity tests (**ASTs**).

Treatment	Anticoccidial	*Eimeria* challenge	Concentration (%)	Dose (ppm)	Inclusion (mg/kg)
T1	No	No			
T2	No	Yes			
T3	Lasalocid	Yes	20	90	450
T4	Maduramycin	Yes	1	6	600
T5	Decoquinate	Yes	6	30	500
T6	Nicarbazin + Semduramicin	Yes	11	66	600
T7	Monensin	Yes	20	110	550
T8	Salinomycin	Yes	12	66	550
T9	Narasin + Nicarbazin	Yes	16	100	625
T10	Nicarbazin	Yes	25	125	500

Schwarzer (2007). These calculations are presented by a specific kind of graphic in meta-analysis denominated Forestplot, can be found each value, pooled effect, weights of each value and confidence interval. In addition, variability between values should be tested to choose fixed or random effects models. Heterogeneity was tested by I-square test. This test evaluates that the differences between measures are random or more than expected. When heterogeneity is significant (I-square > 70−80% or $P < 0.05$), random effect models should be used to calculate pooled effect. When not significant, fixed effect models should be used. This procedure is necessary not to validate the results obtained, but to validate methods used to combine them. Calculations were generally challenge type and compound type. Results were presented by pooled effect, 95% confidence interval, I-square result, and their P-value. Correlations between lesion score and performance variables were calculated with Pearson's correlation coefficient (data was tested for normal distribution by Shapiro-Wilk test ad presented symmetric). The coefficient, the confidence interval 95%, and the P-value were presented.

All analyses were processed in R environmental (R Core Team, 2019), "stats" package (basic package), "ggplot2" (Wickham, 2010) and "meta" (Schwarzer, 2007).

RESULTS

The positive control (T2) was the group that showed the worst performance results and the highest lesion score in four ASTs when compared to the groups that received any of the compounds (T3 to T10). Table 2 shows the pooled effects obtained in meta-analysis models for delta (Δ) BWG at 20 d. A positive effect was observed regardless of the anticoccidial drug used (Overall): 6.52% BWG with confidence interval (**CI**) from 2.21 to 10.80% in challenges with *E. acervulina* and 5.25% BWG with CI from −1.12 to 11.60% in challenges with *E. maxima*. In the *E. acervulina* challenge, the most numerically outstanding compound was decoquinate (**DEC**) ($\Delta = 13.95\%$, CI −0.79 to 28.6%), and the lowest numerical delta value in BWG was maduramycin (**MAD**) ($\Delta = 2.08\%$, CI −10.3 to 14.4%). In the *E. maxima* challenge lasalocid (**LAS**) ($\Delta = 10.35\%$, CI −10.9 to 31.6%) showed the highest delta in BWG, and monensin (**MON**) ($\Delta = 3.10\%$, CI −12.8 to 19.0%) had the lowest delta value in BWG. In both challenges, there was an overlap in the confidence interval, indicating no statistical difference for BWG.

Overall, the use of anticoccidials resulted in a reduction of −2.15% (CI −3.37 to −0.93%) in FCR in challenges with *E. acervulina* and a reduction of −2.13% (CI −3.45 to −0.81%) in challenges with *E. maxima*. The compounds DEC ($\Delta = -4.38\%$, CI −6.15 to −2.61%) and nicarbazin+semduramicin (**NIC**+**SEM**) ($\Delta = -3.37\%$, CI −4.47 to −2.27%) presented lower delta in FCR compared to MON ($\Delta = -0.02\%$, CI −1.38 to 1.34%) and narasin+nicarbazin (**NAR**+**NIC**) ($\Delta = -0.10\%$, CI −1.86 to 1.66%) in challenges with *E. acervulina* (Table 3). In challenge with *E. maxima*, LAS ($\Delta = -5.07\%$, CI −5.29 to −4.85%) showed lower delta in FCR compared to MON ($\Delta = 1.10\%$, CI −2.40 to 4.60%), NAR+NIC ($\Delta = 0.67\%$, CI −4.11 to 5.45%) and nicarbazin (**NIC**) ($\Delta = -3.53\%$, CI −4.37 to −2.69%) (Table 3).

Birds treated with LAS ($\Delta = -55.89\%$, CI −85.44 to −26.34%) or NIC ($\Delta = -33.63\%$, CI −42.19 to −25.08%) presented more reduction in *E. acervulina* lesion scores compared to birds treated with NAR+NIC ($\Delta = -18.70\%$, CI −22.13 to −15.26%) (Table 4). LAS

Table 2. Pooled effects obtained in meta-analysis models for delta body weight gain at 20 d.

Challenge	Treatment	Pooled effects (%)	CI 95% Lower	CI 95% Upper	I² and P-value heterogeneity
E. acervulina	Overall	6.52	2.21	10.8	0%, $P = 1.0$
	Lasalocid	9.08	−2.39	20.5	0%, $P = 0.7$
	Maduramycin	2.08	−10.3	14.4	0%, $P = 0.9$
	Decoquinate	13.95	−0.79	28.6	0%, $P = 0.8$
	Nicarbazin+semduramicin	7.89	−5.20	20.9	0%, $P = 0.7$
	Monensin	3.90	−5.22	13.0	0%, $P = 0.3$
	Salinomycin	5.72	−8.06	19.5	0%, $P = 0.7$
	Narasin+Nicarbazin	7.07	−7.29	21.4	0%, $P = 0.6$
	Nicarbazin	6.57	−5.18	18.3	0%, $P = 0.8$
E. maxima	Overall	5.25	−1.12	11.6	0%, $P = 1.0$
	Lasalocid	10.35	−10.9	31.6	0%, $P = 0.7$
	Maduramycin	5.02	−20.2	30.2	0%, $P = 0.9$
	Decoquinate	9.84	−8.22	27.9	0%, $P = 0.5$
	Nicarbazin+semduramicin	3.47	−15.1	22.1	0%, $P = 0.9$
	Monensin	3.10	−12.8	19.0	0%, $P = 0.9$
	Salinomycin	3.97	−12.5	20.4	0%, $P = 0.7$
	Narasin+Nicarbazin	3.73	−15.6	23.1	0%, $P = 0.8$
	Nicarbazin	4.71	−9.95	19.4	0%, $P = 0.8$

CI: Confidence interval; I2: I-square test percentual and P-value of this. I-square is a heterogeneity test that evaluates if the differences between measures are random or more than expected. When heterogeneity is significant (I-square > 70−0% and/or $P < 0.05$), random effect models were used to calculate pooled effect. When not significant, fixed effect models were used. This procedure is necessary not to validate the results obtained, but to validate methods used to combine them. Significant or not significant differences between pooled effects of each compound can be evaluated by comparing confidence intervals and their intersections. Significant differences are when there is no intersections between CI.

Table 3. Pooled effects obtained in meta-analysis models for delta of feed conversion ratio.

| Challenge | Treatment | Pooled effects (%) | CI 95% | | I^2 and P-value heterogeneity |
			Lower	Upper	
E. acervulina	Overall	−2.15	−3.37	−0.93	100%, $P = 0$
	Lasalocid	−3.01	−5.55	−0.48	99%, $P < 0.01$
	Maduramycin	0.77	−3.10	4.64	100%, $P < 0.01$
	Decoquinate	−4.38	−6.15	−2.61	99%, $P < 0.01$
	Nicarbazin+semduramicin	−3.37	−−4.47	−2.27	98%, $P < 0.01$
	Monensin	−0.02	−1.38	1.34	97%, $P < 0.01$
	Salinomycin	−2.68	−7.45	2.09	100%, $P < 0.01$
	Narasin+Nicarbazin	−0.10	−1.86	1.66	99%, $P < 0.01$
	Nicarbazin	−4.41	−7.38	−1.43	100%, $P < 0.01$
E. maxima	Overall	−2.13	−3.45	−0.81	100%, $P = 0$
	Lasalocid	−5.07	−5.29	−4.85	76%, $P = 0.04$
	Maduramycin	−2.95	−7.18	1.27	100%, $P = 0$
	Decoquinate	−2.19	−8.73	4.36	100%, $P = 0$
	Nicarbazin+semduramicin	−2.63	−4.92	−0.35	100%, $P < 0.01$
	Monensin	1.10	-2.40	4.60	100%, $P < 0.01$
	Salinomycin	−2.42	−7.36	2.52	100%, $P < 0.01$
	Narasin+Nicarbazin	0.67	−4.11	5.45	100%, $P = 0$
	Nicarbazin	−3.53	−4.37	−2.69	97%, $P < 0.01$

CI: Confidence interval; I2: I-square test percentual and P-value of this. I-square is a heterogeneity test that evaluates if the differences between measures are random or more than expected. When heterogeneity is significant (I-square > 70−80% and/or P< 0.05), random effect models were used to calculate pooled effect. When not significant, fixed effect models were used. This procedure is necessary not to validate the results obtained, but to validate methods used to combine them. Significant or not significant differences between pooled effects of each compound can be evaluated by comparing confidence intervals and their intersections. Significant differences are when there is no intersections between CI.

($\Delta = -71.34\%$, CI −78.39 to −64.29%) showed higher reduction in lesion score in challenges with *E. maxima* compared to NIC+SEM ($\Delta = -15.01\%$, CI −32.26 to 2.24%), MAD ($\Delta = -15.20\%$, CI −21.04 to −9.36%), salinomycin ($\Delta = -17.28\%$, CI −30.29 to −4.27%), and NIC ($\Delta = -17.96\%$, CI −24.98 to −10.94%) (Table 4).

Regarding POAA, which is based only in BWG in the 2 control groups (positive and negative) and the test compound, it is possible to observe that DEC ($\Delta = 47.07\%$, CI 25.6 to 68.5%) presented higher percentage of OAA compared to MAD ($\Delta = 6.60\%$, CI −6.55 to 19.7%) and MON ($\Delta = 7.06\%$, CI −11.4 to 25.5%) in *E. acervulina* challenged birds. In *E. maxima* challenged birds, DEC showed higher POAA compared to NIC+SEM, salinomycin and NIC (Table 5). The analysis of POAA classification in R/PR/S in the challenge with *E. maxima* revealed sensitivity to decoquinate, partial resistance to lasalocid, and resistance to the remaining test compounds. All compounds were classified as resistant in the challenge with *E. acervulina*. Figure 1 shows the splitting of POAA results in AST 1 and 4, when the challenge was with *E. maxima*. Details

Table 4. Pooled effects obtained in meta-analysis models for delta lesion score.

| Challenge | Treatment | Pooled effects (%) | CI 95% | | I^2 and P-value heterogeneity |
			Lower	Upper	
E. acervulina	Overall	−117.83	−117.89	−177.77	100%, $P < 0.001$
	Lasalocid	−55.89	−85.44	−26.34	100%, $P < 0.001$
	Maduramycin	−58.48	−147.40	30.43	100%, $P < 0.001$
	Decoquinate	−503.27	−1252.84	246.30	100%, $P < 0.001$
	Nicarbazin+semduramicin	−46.34	−105.78	13.11	100%, $P < 0.001$
	Monensin	−20.59	−31.56	−9.63	100%, $P < 0.001$
	Salinomycin	−29.23	−46.42	−12.03	100%, $P < 0.001$
	Narasin+Nicarbazin	−18.70	−22.13	−15.26	100%, $P < 0.001$
	Nicarbazin	−33.63	−42.19	−25.08	100%, $P < 0.001$
E. maxima	Overall	−62.91	−144.41	18.59	100%, $P < 0.001$
	Lasalocid	−71.34	−78.39	−64.29	100%, $P < 0.001$
	Maduramycin	−15.20	−21.04	−9.36	100%, $P < 0.001$
	Decoquinate	−294.16	−795.60	207.27	100%, $P < 0.001$
	Nicarbazin+semduramicin	−15.01	−32.26	2.24	100%, $P < 0.001$
	Monensin	−38.48	−106.79	29.82	100%, $P < 0.001$
	Salinomycin	−17.28	−30.29	−4.27	100%, $P < 0.001$
	Narasin+Nicarbazin	−33.86	−80.34	12.62	100%, $P < 0.001$
	Nicarbazin	−17.96	−24.98	−10.94	100%, $P < 0.001$

CI: Confidence interval; I2: I-square test percentual and P-value of this. I-square is a heterogeneity test that evaluates if the differences between measures are random or more than expected. When heterogeneity is significant (I-square > 70−80% and/or $P < 0.05$), random effect models were used to calculate pooled effect. When not significant, fixed effect models were used. This procedure is necessary not to validate the results obtained, but to validate methods used to combine them. Significant or not significant differences between pooled effects of each compound can be evaluated by comparing confidence intervals and their intersections. Significant differences are when there is no intersections between CI.

Table 5. Pooled effects obtained in meta-analysis models for percentage of optimal anticoccidial activity (POAA).

Challenge	Treatment	Pooled effects (%)	Classification R/PR/S	CI 95% Lower	CI 95% Upper	I² and P-value heterogeneity
E. acervulina	Overall	22.45	R	13.8	31.1	74%, $P < 0.01$
	Lasalocid	33.09	R	−1.27	67.4	86%, $P < 0.01$
	Maduramycin	6.60	R	−6.55	19.7	0%, $P = 0$
	Decoquinate	47.07	R	25.6	68.5	68%, $P = 0$
	Nicarbazin+semduramicin	18.62	R	6.3	30.9	0%, $P = 0$
	Monensin	7.06	R	−11.4	25.5	48%, $P = 0$
	Salinomycin	21.63	R	−4.04	47.3	74%, $P = 0$
	Narasin+Nicarbazin	18.80	R	6.30	31.3	0%, $P = 0$
	Nicarbazin	23.72	R	0.21	47.2	70%, $P = 0$
E. maxima	Overall	47.27	R	39.2	55.3	92%, $P < 0.01$
	Lasalocid	65.9	PR	36.7	95.1	92%, $P = 0$
	Maduramycin	40.2	R	3.83	76.6	95%, $P = 0$
	Decoquinate	81.8	S	53.3	110.3	91%, $P = 0$
	Nicarbazin+semduramicin	34.0	R	25.6	42.4	27%, $P = 0$
	Monensin	24.6	R	−−19.1	68.4	96%, $P = 0$
	Salinomycin	41.6	R	35.1	48.2	0%, $P = 0$
	Narasin+Nicarbazin	49.4	R	26.8	72.1	87%, $P = 0$
	Nicarbazin	41.8	R	35.7	47.9	0%, $P = 0$

CI: Confidence interval; I2: I-square test percentual and *P*-value of this. I-square is a heterogeneity test that evaluates if the differences between measures are random or more than expected. When heterogeneity is significant (I-square > 70−80% and/or $P < 0.05$), random effect models were used to calculate pooled effect. When not significant, fixed effect models were used. This procedure is necessary not to validate the results obtained, but to validate methods used to combine them. Significant or not significant differences between pooled effects of each compound can be evaluated by comparing confidence intervals and their intersections. Significant differences are when there is no intersections between CI.

Abbreviations: PR, Partially Resistant; R, Resistant; S, Sensitive.

of all parameters can be found in the supplementary material as Forestplot graphics.

The correlation analysis between lesion score and the variables FCR, BWG, and POAA included treatments T3 to T10. It combined the results of the 4 ASTs and separated by type of challenge (Table 6). No significant correlation between ΔFCR and lesion score was observed. A negative correlation between ΔBWG and LS was observed ($r = −36$) overall and in the challenge with *E. acervulina* ($r = −0.59$), that is, the lower the BWG, the higher the LS (Figure 2). The POAA showed a negative correlation with LS in the overall analysis ($r = −0.42$), and in the challenges with *E. maxima* ($r = −60$) and with *E. acervulina* ($r = −0.43$).

DISCUSSION

The last study of this category carried out in Brazil was conducted by Mcdougald et al., 1987. Sixty samples of *Eimeria* (a mix of several species − not specified the dose of the inoculum) were isolated and submitted to an extensive test with the 7 compounds most commonly used at that time (monensin, narasin, salinomycin, maduramycin, clopidol, amprolium, and nicarbazin). Although a direct comparison between studies does not seem adequate, the difference in lesion score and BWG between the treated group and the nonmedicated and infected control group reveals a significant change in the last 30 yr. The assessed compounds reduced lesion scores
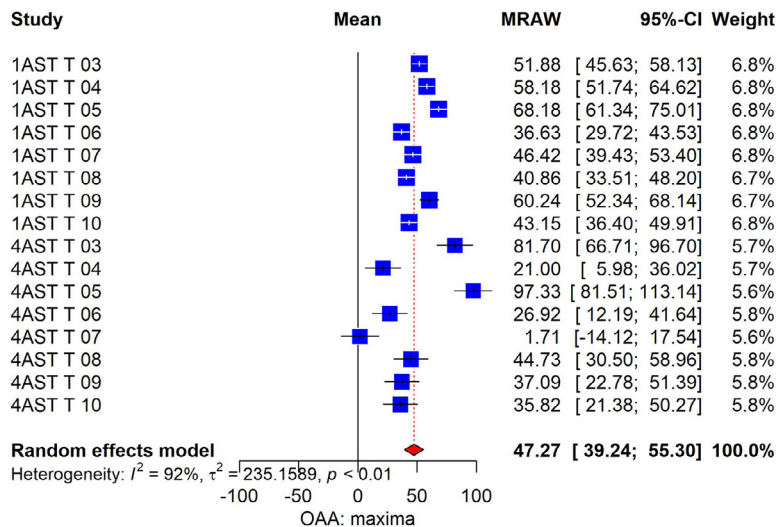
Figure 1. Forestplot of meta-analysis model of optimum anticoccidial activity (POAA) with different treatments in ASTs 1 and 4 (challenge with *E. maxima*). T03: lasalocid, T04: maduramycin T05: decoquinate, T06: nicarbazin+semduramicin, T07: monensin, T08: salinomycin, T09: narasin+nicarbazin, T 10: nicarbazin. Blue square are the mean of POAA in each experimental group and each AST; red diamond represents pooled effect of all experimental groups considered.

Table 6. Correlation coefficient between lesion scores and other variables.

| Variable | Challenge | Correlation coefficient | Confidence interval for coefficient | | P-value |
			Lower	Upper	
Delta feed conversion 1−20 d	Overall	−0.07	−0.41	0.28	0.698
	E. acervulina	0.11	−0.40	0.58	0.674
	E. maxima	−0.27	−0.67	0.27	0.321
Delta body weight gain 1−20 d	Overall	−0.36	−0.63	−0.02	0.041
	E. acervulina	−0.59	−0.84	−0.13	0.017
	E. maxima	−0.10	−0.57	0.42	0.715
POAA	Overall	−0.42	−0.67	−0.09	0.016
	E. acervulina	−0.43	−0.76	0.09	0.098
	E. maxima	−0.60	−0.84	−0.14	0.015

by 52% on average and improved weight gain by 77.7% compared to the control group (Mcdougald et al., 1987). In the present study, the overall average of the used compounds resulted in an improvement of 6.52% in weight gain in challenge with *E. acervulina* and of 5.52% in challenge with *E. maxima*. Similar results were found by Conway et al., 2001 in 2 studies. The investigators used salinomycin, monensin, and lasalocid in one of

these experiments, and nicarbazin, narasin+nicarbazin, and zoalene in the other study. Arabkhazaeli et al., 2013 also evaluated salinomycin, amprolium+ethopabate, and diclazuril and reported similar results.

Considering FCR as a strong indicator of drug sensitivity, MON and NAR+NIC presented lower delta compared to DEC in challenge with *E. acervulina* and compared to LAS in challenge with *E. maxima*. This
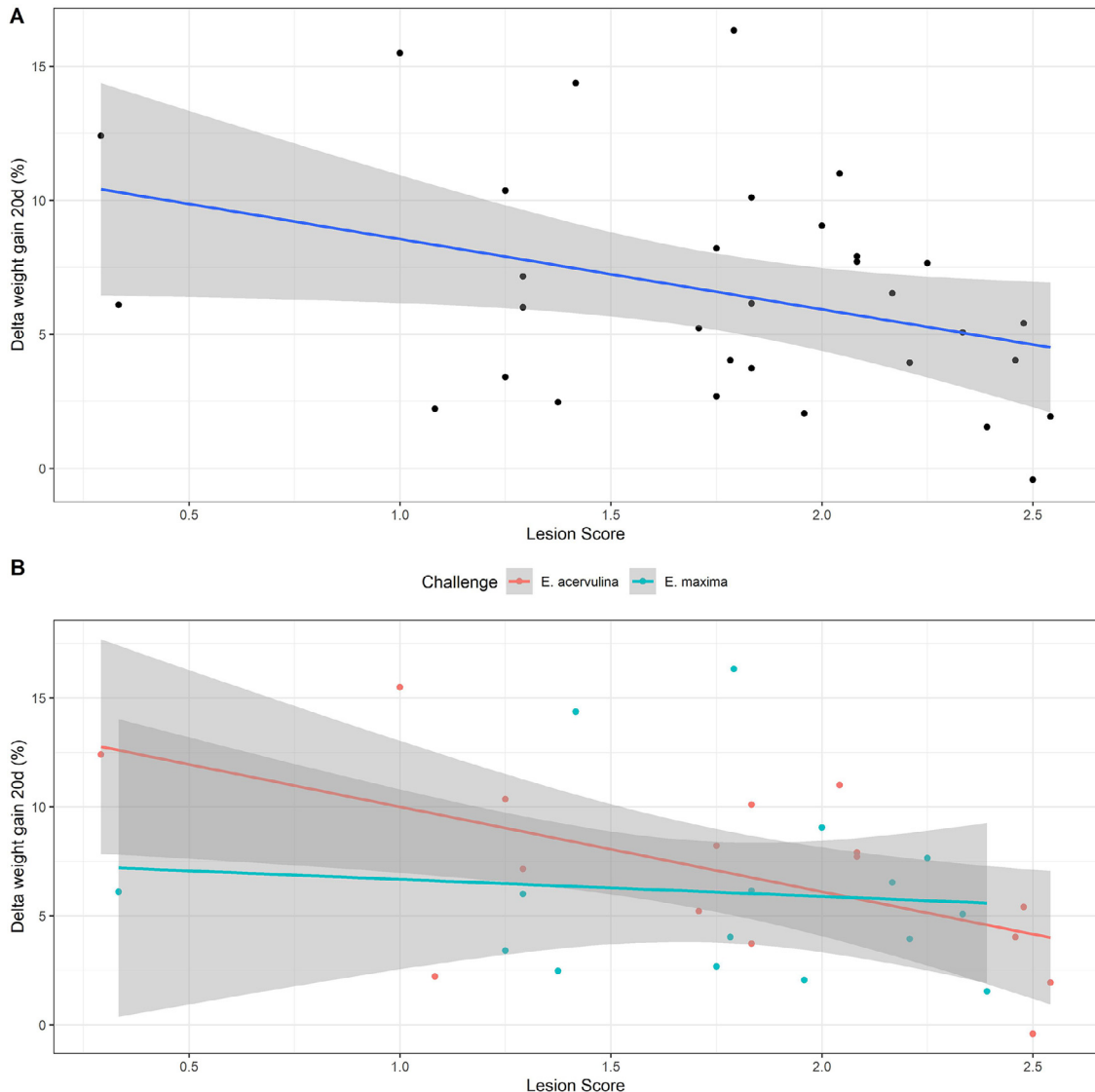
Figure 2. Scatterplot of correlation between lesion score and delta weight gain, regardless of challenge (A) and separating challenges with *E. acervulina* and *E. maxima* (B).

does not mean that these compounds have completely lost their efficacy, since analysis of the confidence interval reveals different effect possibilities, which may be influenced by different field conditions. An interesting point is that even after 50 yr of use, monensin remains an important tool in coccidiosis control programs (Chapman et al., 2010). In general, a partial loss of efficacy combined with immunity acquisition explains the continuity of ionophore efficacy in the field (Chapman et al., 2010).

Compounds showing considerable effects on BWG, FCR, POAA, and lesion scores independent of the main challenge were decoquinate and lasalocid. One hypothesis for this finding would be that these drugs would have "rested" due to the low frequency of use in anticoccidial programs and that their sensitivity was restored. Chapman and Jeffers, 2015 observed this effect of restoration of sensitivity with salinomycin after 5 flocks were reared under different drug programs and vaccine use. It is important to point out that, in our experiments, oocysts were not propagated in live chickens but were filtered and used directly as inoculum, which minimizes oocyst selection. However, the doses used are considered high, and this does not reflect the magnitude of field challenges (Chapman, 1999).

Another relevant fact of this study is that treatment with nicarbazin (T10) apparently was not the most effective in ASTs. This caught our attention, because it is very common for treatment with nicarbazin to stand out in relation to other anticoccidials in similar assessments conducted in the United States and Europe (Mathis et al., 1984; Mcdougald et al., 1986; Bafundo et al., 2008). Although nicarbazin is known to be the most effective compound in coccidiosis control, this might be changing. The reason may be that Brazilian anticoccidial programs have continuously used pure nicarbazin or associated with another ionophore without any rotation. The mechanisms why Eimeria spp. develop resistance against nicarbazin are still poorly understood (Chapman, 1997). However, this issue deserves a deeper understanding, by increasing the number of ASTs in other Brazilian regions to determine how widely this occurs.

Many factors may interfere in the pathogenicity and sensitivity profile of drugs against Eimeria spp., such as region, previous exposure to other drugs, and use of the same drug for a long period (Tan et al., 2017). However, approaches used to detect resistance or virulence factors in several bacteria (Fluit et al., 2001) are not yet available for Eimeria (Blake et al., 2020b). Therefore, even with limiting factors, such as cost and slow procedure, in vivo experiments are the only way to estimate the sensitivity profile of Eimeria spp. to anticoccidials (Peek and Landman, 2011). This study's differential is that multiplication in live birds was not necessary because of the large volume of feces collected (5−7 kg). Literature suggests oocysts multiplication in live birds could result in selection of non-relevant coccidia (Peek and Landman, 2011). Thus, we can state that the results obtained

with this methodology are closer to the reality in the field.

Although fighting resistance against anticoccidials is a difficult task, shuttle programs and rotation of compounds are approaches that help prevent or postpone its emergence (Quiroz-Castañeda and Dantán-González, 2015). In shuttle programs, different drugs are alternated in a same flock, whereas in rotation programs, different drugs are alternated after one or several seasonal or rearing cycles (Gussem, 2007). Strictly speaking, rotating between a monovalent ionophore and another may be considered rotation. However, considering that cross-resistance may happen within the same class of ionophores (Weppelman et al., 1977), the relevance of this type of rotation could be questioned (Gussem, 2007). The rotation of compounds − when rationally used − helps restore available drug efficacy since it promotes a rest period between compound usage (Chapman and Jeffers, 2015). Another means of restoring drug efficacy is the use of live vaccines, because they change the oocyst population on the litter with sensitive vaccine strains (Snyder et al., 2021).

Of all the parameters used in this study, the POAA seems to be the least accurate for sensitivity assessment to anticoccidials. Categorization into R, PR, and S does not appear to be a good indicator for understanding the differences in sensitivity level between different inocula. Perhaps for this reason, few scientific studies have adopted this form of description by category of sensitivity to Eimeria spp., differently from what is usually done for bacteria in antibiograms.

This study confirmed and identified different sensitivity levels of E. acervulina and E. maxima to these eight compounds in different Brazilian regions, based mainly in FCR. Sensitivity tests to anticoccidials in wire cages permit a good diagnosis of Eimeria sensitivity. They can substantiate making the decision to change or continue the use of more effective compounds. Even if some compounds do not further lose their effectiveness completely, frequent change − based on sensitivity studies − can identify opportunities for performance and economic gains.

DISCLOSURES

The authors declare that there is no conflict of interests regarding the publication of this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi: https://doi.org/10.1016/j.psj.2021.101233.

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